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(54) **Title:** VECTOR FOR LACTIC ACID BACTERIA AND METHOD FOR EXPRESSING FERRITIN IN THE LACTIC BACTERIA

(57) **Abstract:** Disclosed is an integration vector for use in lactic acid bacteria. More particularly, disclosed are an integration vector for use in lactic acid bacteria, pJC2, which is integrated into chromosomal DNA of a lactic acid bacterium, thereby capable of being successfully replicated in the lactic acid bacterium; an integration vector, pJC7F, capable of successfully expressing human-derived ferritin gene in lactic acid bacteria; and lactic acid bacteria transformed with pJC2 or pJC7F. The vectors pJC2 and pJC7F are applicable as food grade additives. Using those vectors, thus, it is possible to successfully express a ferritin protein in transformed lactic acid bacteria, the ferritin protein being used as an iron agent.

VECTOR FOR LACTIC ACID BACTERIA AND METHOD FOR EXPRESSING FERRITIN IN THE LACTIC ACID BACTERIA

Technical Field

The present invention relates to a vector for use in lactic acid bacteria and a method for expressing ferritin in lactic acid bacteria. More particularly, the invention relates to a vector can be successfully replicated in lactic acid bacteria, as well as for use in easily screening transformed lactic acid bacterium of which chromosomal DNA contains the vector integrated thereto, owing to a certain selection marker gene carried therein, and a method for massively expressing ferritin in lactic acid bacteria by using a vector containing a ferritin gene.

Background Art

Lactic acid bacteria are microorganisms widely distributed in nature, and which produce lactic acid by anaerobic utilization of carbohydrates. Since lactic acid bacteria inhibit growth of putrefying microorganisms which grow well in neutral and alkali conditions, they have been utilized as an important means of food preservation in both the East and West. Especially, lactic acid bacteria have been used for processing milk, dairy goods, meat products, vegetables fermented in salt, and other diverse salted products. Such lactic acid bacteria produce organic acids such as lactic

acid and acetic acid, thereby serving as a bactericide against many microorganisms in the intestines. Theretore, lactic acid bacteria decrease pH of foods, while improving the shelf-life of foods. Recently, lactic acid bacteria have attracted attention in the food industry. It is known that lactic acid bacteria play an important role in maintaining health of the stomach and intestines. In addition, it was reported that lactic acid bacteria are capable of improving health upon directly ingesting them (Matsuzaki *et al.*, *Int. J. Food. Microbiol.*, 41: 133-140; Bengmart, Gut, 42: 2-7; Gilliard, *FEMS Microbiol. Rev.*, 87: 175-188; Perdigon *et al.*, *J. Dairy Sci.*, 70: 919-926, 1987; and Tejada-Simon *et al.*, *J. Food. Prot.*, 62: 1435-1444, 1999).

Current reports prove effects of lactic acid bacteria on stabilization of intestinal microflora, prevention of harmful bacteria colonization in the intestines, and treatment of intestinal diseases, etc. Accordingly, the use of lactic acid bacteria as a functional material such as a food additive, as well as being employed as a food itself, has attracted worldwide attention. Furthermore, commercialization of lactic acid bacteria is underway. Especially, since the time when Elie Metchinikoff conducted scientific studies on health benefits of fermented milk, many studies have been conducted to find possible benefits of lactic acid bacteria, including anticancer activity, control of serum cholesterol, immune-regulation, and improvement of local and systemic immunity. In addition, *Lactobacillus acidophilus* and *Lactobacillus bifidum* are now available as food additives, to improve immune functions of newborn infants and young children with deteriorated immunity.

Thanks to recent biotechnologies, active research to develop lactic acid bacteria with novel functions is underway worldwide. Lactic acid bacteria have been

granted GRAS (Generally Recognized As Safe) status, and have a mechanism of secreting proteins extracellularly. And, because of low titer of extracellular proteinases, unlike *Bacillus* species, lactic acid bacteria are useful for producing foreign proteins. Using these advantages, intensive studies are underway, in fields of delivery of enzymes, antibiotics, and vaccines, etc. to epithelial cells of the intestines by utilizing lactic acid bacteria. In the future, lactic acid bacteria newly developed for those purposes will be of great value in economic, social and medical aspects.

Therefore, in order to raise industrial application values of lactic acid bacteria, it is effective way to improve the strains using genetic engineering techniques. Worldwide research is focused on finding ways to express foreign proteins in the lactic acid bacteria in a massive manner, for example, by the use of high-copy number plasmid, strong constitutive promoter, and regulated promoter, etc. (Jos M.B.M. Van Der Vossen, Daniel Van Der Lelie, and Gerard Venema, *Appl. Environ. Microbiol.* 53: 2452-2457).

However, once such industrially beneficial lactic acid bacteria are improved by means of genetic engineering techniques, it is an important matter to consider whether the improved vector is stably present within the transformed lactic acid bacteria, while stably retaining its genetic character.

In improving lactic acid bacteria by means of genetic engineering techniques or developing new goods using the improved lactic acid bacteria, "safety" is an essential factor to consider in improving bacteria applicable to food industry. That is, a safety-guaranteed cloning vector for use in foods, which is able to be integrated into chromosomal DNA of lactic acid bacteria, is demanded.

As a part of such development of cloning vector for use in foods, a nonreplicatve plasmid was developed, but the plasmid has a disadvantage of very low transformation efficiency (Walker, D. C and T.R. Klaenhammer, *J. Bacteriol.*, 176: 5330-5340, 1994; Emmanuelle M. *et al.*, *J. Bacteriol.*, 178: 931-935, 1995).

Now, researchers of U.S, Europe, etc. who are the world's leading groups studying lactic acid bacteria, are directed to vaccine development using lactic acid bacteria, delivery of hormone agents to the intestines by the help of lactic acid bacteria, and development of an integration vector for delivery, though such research is still in the beginning stage.

Further, most of lactic acid bacteria secrete β-galactosidase, and frequently generate revertants upon use of an antibiotic resistance gene as a selection marker. For those reasons, there is a difficulty in screening only transformants on media containing X-gal and antibiotics. Accordingly, attempts to find an easier way of screening transformants are continuing.

Meanwhile, ferritin protein is an iron-storage protein and a spherical protein bearing iron ions in the center, and is widely distributed in liver, spleen and bone marrow. Ferritin binds iron in cells as a reservoir of iron, thereby protecting cells from toxicity of iron, while inhibiting reaction of iron with oxygen. Accordingly, ferritin performs important roles of protection of proteins and lipids in cells. Meanwhile, deficiency of iron causes many problems to humans. Such deficiency frequently occurs in infants, pregnant women, juveniles and teenagers. Only the ingestion of foods containing iron is not enough to achieve treatment and prevention of iron

deficiency, so there is a need for iron agents with high bioavailability, containing 10 to 50 mg iron, which is the recommended amount for daily ingestion.

Iron agents now commercially available in Korea are agents for treatment of anemia, and are made of ferritin extracts obtained from spleens of horse or cattle, the extracts being imported. However, the ferritin component extracted from animals consists of amino acids different from those of humans. For this reason, upon absorption into the body by administering such a ferritin component to humans, the efficiency of functions and effects is doubtful.

In Korea, about 7 hundred million dollars worth of iron agents were sold in 1999. As for blood-forming medicines, the world market amounts to 26 hundred million dollars per year. In Korea, about 92 % of the domestic iron agents were, unfortunately, judged to be unfit for humans, so the iron agents for use as a food additive and as a medicine entirely depend on imports. In addition, epidemic of the mad cow diseases caused consumers to show great distrust for current iron agents comprising iron proteins extracted from livestock. If new iron agents are developed to substitute for the existing agents, they will meet considerable demand in the Korean domestic market. Therefore, substitute iron proteins are demanded without delay.

Inside and outside of Korea, research and development of iron agents leads to an attempt to commercialize erythropoietin by transforming mammalian cells to make erythropoietin produce, a hormone increasing production of red blood cells. However, since erythropoietin is just a hormone, not a functional protein, and is expensive, the application range and demand are very limited. On the other hand, if ferritin is employed as an iron agent, there are advantages of high efficacy, and low-cost. Thus,

development of a production system for expressing ferritin in lactic acid bacteria is demanded.

The inventors developed a vector for use in lactic acid bacteria, capable of expressing ferritin within transformed lactic acid bacteria, in an effective manner, while stably retaining its genetic character and being applicable as a food grade additive. Particularly, the inventors developed a vector for use in lactic acid bacteria, capable of successfully expressing human-derived ferritin, and found that transformation of lactic acid bacteria with the vector allows successful expression of ferritin therein.

Disclosure of the Invention

Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to provide a method for expressing ferritin in lactic acid bacteria transformed with a vector containing a ferritin gene.

It is another object of the present invention to provide a food additive or pharmaceutical agent containing ferritin expressed by the above method.

It is yet another object of the present invention to provide a vector which can be successfully replicated in lactic acid bacteria, as well as for use in easily screening transformed lactic acid bacteria of which chromosomal DNA contains the vector integrated thereto, owing to a certain selection marker gene carried in the vector.

In accordance with one aspect of the present invention, the above and other objects can be accomplished by the provision of a method for expressing ferritin in

lactic acid bacteria transformed with a vector containing a ferritin gene. Preferably, the vector containing the ferritin gene is a vector for use in lactic acid bacteria, containing an origin of replication. The vector containing the ferritin gene is more preferably a vector containing an origin of replication derived from a Gram-negative bacterium. Furthermore, the vector may contain an insertion sequence, a *cel*A gene and an antibiotic resistance gene.

In accordance with another aspect of the present invention, there is provided an integration vector for use in lactic acid bacteria, containing a *cel*A gene for determining whether the vector is integrated into chromosomal DNA of a lactic acid bacterium and an insertion sequence.

In accordance with yet another aspect of the present invention, there is provided an integration vector for use in lactic acid bacteria, containing an origin of replication derived from a Gram-negative bacterium, an erythromycin resistance gene, nisin A promoter, a *cel*A gene derived from *Clostridium thermocellum*, IS1223, a ferritin gene, a gene encoding His · Tag sequence, and a cp25 promoter represented by SEQ ID NO: 3.

Hereinafter, the invention is described in detail.

The invention is directed to expression of ferritin in lactic acid bacteria. To this, a lactic acid bacterium is transformed with a vector containing a ferritin gene. The vector according to the invention contains an origin of replication derived from a Gram-negative bacterium, not a Gram-positive bacterium. As a result, the origin alone fails to maintain the vector in a stable manner within a Gram-positive lactic acid

bacterium. Accordingly, owing to an insertion sequence, the vector can be integrated into chromosomal DNA of the lactic acid bacterium which is a Gram-positive bacterium.

The vector according to the invention may be an integration vector for use in lactic acid bacteria, containing a *cel*A gene for determining whether the vector is integrated into chromosomal DNA of a lactic acid bacterium, and an insertion sequence.

Most of lactic acid bacteria secrete β-galactosidase, and frequently generate revertants upon use of an antibiotic resistance gene as a selection marker. For those reasons, there is a difficulty in screening only transformants on media containing X-gal and antibiotics. Thus, for accurate and easy screening of transformants, the vector containing the *cel*A gene is preferably employed.

As for the insertion sequence inserted into the integration vector according to the invention, all known insertion sequences are available. Especially, the insertion sequence is preferably IS1223, IS481, IS100 and IS903, more preferably, IS1223. IS1223 is an insertion sequence derived from *Lactobacillus johnsonii*. It was reported that IS1223 mediates random integration and multi-copy insertion (Walker, D. C. and T. R. Klaenhammer, *J. Bacteriol.*, 176: 5330-5340, 1994).

Though the promoter for the *cel*A gene in the integration vector for use in lactic acid bacteria may be any promoters for use in lactic acid bacteria, without special limitation, a nisin A promoter is preferable.

The vector according to the invention may contain a known antibiotic resistance gene which is commonly used. Especially, an erythromycin or

chloramphenicol resistance gene is preferable, and the erythromycin resistance gene is more preferable.

Particularly, the invention is directed to pJC2, an integration vector for use in lactic acid bacteria, containing an origin of replication which is derived from a Gram-negative bacterium, a nisin A promoter, a *cel*A gene derived from *Clostridium thermocellum*, and IS1223.

To construct pJC2, first, pTRK327 containing an erythromycin resistance gene (em^r), IS1223 and an origin of replication from a Gram-negative bacterium is inserted with a *lac*Z gene and a *nis*A promoter from pLB1, thereby constructing pJC1. A *cel*A gene from pTvec/*cel*A, a vector which was constructed by inserting a *cel*A gene into T vector (Promega) is inserted into pJC1, thereby constructing pJC2-1. From pJC2-1 a chloramphenicol resistance gene is removed, thereby constructing pJC2-2. From pJC2-2, many restriction sites are removed, thereby constructing pJC2.

The vector according to the invention contains a ferritin gene as a target gene. But, as for the target gene capable of being inserted into the integration vector according to the invention, any genes expressing proteins useful to humans including a gene encoding an antihypertensive protein or a gene encoding a peptide for vaccination, may be inserted.

As for the promoter for the target gene capable of being inserted into the integration vector according to the invention, a cp25 promoter, and promoters P21, P23 and P59 are preferable. More preferably, the cp25 promoter represented by SEQ ID NO: 3 may be used.

The integration vector according to the invention may contain a His · Tag sequence which is inserted between a promoter for the target gene and a gene encoding the target protein.

An expressed target gene can be easily purified, by inserting a certain nucleotide sequence into the integration vector. As for the certain nucleotide sequence capable of being inserted into the integration vector, a His · Tag sequence, a CBD sequence, and a S · Tag sequence is preferable. More preferably, the His · Tag sequence may be used.

In the invention, the lactic acid bacteria transformed with the vector containing the ferritin gene was screened on an agar plate containing erythromycin and a substrate for cellulase. As for the substrate, cellulose is preferable. In the invention, cellomixTM agar plate which is commercially available, was used.

The method for transforming *E. coli* and lactic acid bacteria with the vector according to the invention is preferably electroporation or calcium chloride method. More preferably, electroporation is used.

The lactic acid bacteria capable of being transformed with the integration vector, are preferably *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus cassei* or *Lactobacillus amylovorus*. More preferably, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus bulgaricus*, and *Lactobacillus plantarum* are used.

Integration of the integration vector of the invention into chromosomal DNA of a lactic acid bacterium may be determined by the presence of a 1.4 kb band after amplifying the *cel*A gene inserted into the vector by PCR. In addition, the integration

may be confirmed by performing Southern blotting of the chromosomal DNA of the transformant, using the amplified *cel*A gene as a probe. These tests demonstrated that pJC2 was integrated into the chromosomal DNA of *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus bulgaricus*, and *Lactobacillus plantarum*, respectively.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows a construction process of pJC2, an integration vector for use in lactic acid bacteria, according to a preferred embodiment of the invention;

Fig. 2 is a cleavage map of pJC2, an integration vector for use in lactic acid bacteria;

Fig. 3 shows cellulase halos formed on a cellomixTM plate by a transformant transformed with pJC2, an integration vector for use in lactic acid bacteria;

Fig. 4 shows a result of PCR amplifying a *cel*A gene from a transformant transformed with pJC2, an integration vector for use in lactic acid bacteria, wherein:

lane 1; λ/*Hind*III marker;

lane 2, a result of PCR performed using chromosomal DNA from a non-transformed lactic acid bacterium as a template;

lane 3, a result of PCR performed using chromosomal DNA from a lactic acid

bacterium transformed with pJC2 as a template; and

lane 4, a positive control, a result of PCR performed using pJC2 alone as a template;

Fig. 5 shows a result of Southern blot performed using chromosomal DNA from a lactic acid bacterium transformed with integration vector pJC2 and a *cel*A gene as a probe, wherein:

lane 1, a positive control, a Southern blot of pJC2 digested with *Eco*R1;

lane 2, a Southern blot of chromosomal DNA from *Lactobacillus gasseri* digested with *Kpn*I;

lane 3, a Southern blot of chromosomal DNA from *Lactobacillus gasseri* digested with *Eco*RI;

lane 4, a Southern blot of chromosomal DNA from *Lactobacillus johnsonii* digested with *Kpn*I;

lane 5, a Southern blot of chromosomal DNA from *Lactobacillus johnsonii* digested with *Eco*RI;

lane 6, a negative control, a Southern blot of chromosomal DNA from *Lactobacillus gasseri*;

lane 7, a positive control, a Southern blot of pJC2 digested with *Eco*R1;

lane 8, a Southern blot of chromosomal DNA from *Lactobacillus bulgaricus* digested with *Kpn*I; and

lane 9, a Southern blot of chromosomal DNA from *Lactobacillus bulgaricus* digested with *Eco*RI;

Fig. 6 is a cleavage map of pJC7F, an integration vector constructed by

inserting a promoter and a ferritin gene into vector pJC2;

Fig. 7 shows a result of PCR amplifying a cp25 promoter and a ferritin gene, using chromosomal DNA from a transformant transformed with pJC7F, an integration vector for use in lactic acid bacteria, as a template, wherein:

lane 1, a negative control, a result of PCR performed using chromosomal DNA from a non-transformed lactic acid bacterium as a template;

lanes 2 and 3, results of PCR performed using chromosomal DNA from *Lactobacillus bulgaricus* transformed with pJC7F as a template;

lane 4, λ/*Hind*III marker; and

lane 5, a positive control, a result of PCR performed using pJC7F alone as a template;

Fig. 8 shows a result of SDS-PAGE of a ferritin protein expressed in the transformant, the protein being partially purified by using a His · Tag column, wherein:

lane 1, MBI protein marker;

lanes 2 to 7, fractions of iron proteins purified by the His · Tag column; and

lane 8, cell extract of bacteria expressing iron protein.

Best Mode for Carrying Out the Invention

Hereinafter, the present invention will be described in detail, in conjunction with various examples. These examples are provided only for illustrative purposes, and the present invention is not to be construed as being limited to those examples.

Example 1

Construction of pJC2, an integration vector for use in lactic acid bacteria

Fig. 1 schematically illustrates a construction process of pJC2, an integration vector of the invention. First constructed was a pTRK327 (North Carolina State University) containing an erythromycin resistance gene (em^r), a tetracyclin resistance gene (tet^r), a chloramphenicol resistance gene (cm^r), IS1223, and a replication origin of a Gram-negative bacterium. IS1223 had been isolated from *Lactobacillus johnsonii* NCK88.

The plasmid pTRK327 was employed to construct a recombinant vector pJC1. A 3.3 kb *Bam*HI-*Bgl*II fragment containing *lacZ* and a *nisA* promoter was removed from a vector pLB1 (Seoul University) and inserted into the pTRK327, thus constructed pJC1. The *lacZ* gene and *nisA* promoter had been isolated from *Lactococcus lactis*. The recombinant vector, pJC1 thus constructed was used to transform *E. coli* XL1-Blue (NEB) and *Lactobacillus gasseri* ATCC 33323, respectively. The transformed *E. coli* and *Lactobacillus gasseri* formed blue colonies on a X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plate.

From a vector, pTvec/*cel*A, the *cel*A gene of a 1.4 kb *Apa*I-*Sal*I segment, derived from *Clostridium thermocellum*, was removed, and the *cel*A gene was inserted into the recombinant vector pJC1. Therefore, a recombinant vector pJC2-1, 9.6 kb long, and containing a second selection marker, *cel*A, was constructed. Using *Pvu*II and *Ava*I, cm^r was removed from the pJC2-1, thus constructed a 7.8 kb recombinant vector, pJC2-2. Using *Nco*I, many restriction sites were removed from pJC2-2, and the ends were ligated using a blunt ligation enzyme (Takara Shuzo Co., Ltd., Japan).

Therefore, an integration vector for use in lactic acid bacteria, pJC2, 7.6 kb long, and containing an origin of replication derived from a Gram-negative bacterium ((-) origin), a *nis*A promoter, a *cel*A gene, an erythromycin resistance gene (em^r) and IS1223, was constructed. The cleavage map of pJC2 is shown in Fig. 2.

Example 2

Transformation using an integration vector, pJC2 and transformant screening

With the integration vector pJC2 constructed as in Example 1, *Lactobacillus gasseri* ATCC 33323 and *E. coli* XL1-Blue (NEB) were respectively transformed using an electroporation method. For transformation, competent *Lactobacillus* cells were prepared by a modified 'Holo and Nes method' (Holo, H., and I. F. Nes, *Appl. Environ. Microbiol.*, 56: 1890-1896). A *Lactobacillus* strain was inoculated into 5 ml MRS medium and pre-cultured overnight. The cell culture was subcultured in 100 ml MRS medium (Difco) containing 0.2 % glycine and 0.5 M sucrose until absorbency at 600 nm reached to 0.5 - 0.7. The cultured cells were centrifuged at 4℃ and at 6,000 rpm for 10 min. After centrifugation the cells were collected and suspended in 20 ml cold distilled water, followed by centrifuging under the above conditions. The collected cells were resuspended in distilled water as in the above, followed by centrifuging. The collected cells were resuspended in a cold 1X SMEB buffer solution consisting of 78 mM sucrose and 0.29 mM $MgCl_2$, followed by centrifuging at 4℃ and 6,000 rpm for 10 min. After removing the supernatant, the cell pellet was suspended in one volume of the SMEB buffer solution, relative to the cell pellet volume, and stored at -70℃ until use for transformation. On the other hand, competent *E. coli* cells for

transformation were prepared by a known method (Dower, W.J., J. F. Miller, and C.W.Ragsdale. *Nucleic Acids Res.*, 16:6127-6145, 1988).

Using the competent cells prepared as in the above, electroporation was performed. 40 ㎕ of the competent *E. coli* cells and 100 to 200 ng of the plasmid DNA were mixed in a 0.2 cm cuvette (Bio-Rad), and subjected to an electrical pulse of 2.5 kV, 25 μF by means of Gene pulser unit (Bio-Rad). The transformants thus constructed were incubated at 37℃ for 1 hr, in SOC medium consisting of 0.5 % yeast extract, 2 % tryptone, 0.05 % sodium chloride, 250 mM potassium chloride, 2 M magnesium chloride, and 1 M glucose.

E. coli was cultured in LB broth (Difco). The transformed *E. coli* cells were screened by incubating at 37℃ on LB agar plates (1.5 % agar) containing cellomixTM (RNA Inc., Seoul, Korea), 200 ㎍/㎖ erythromycin, 10 ㎍/㎖ chloramphenicol and 100 ㎍/㎖ ampicillin. Transformation of the competent *Lactobacillus* cells was performed by the same method as in the above *E. coli* transformation.

Lactobacillus gasseri was cultured in MRS broth (Difco). The transformed *Lactobacillus gasseri* cells were screened by incubating at 37℃ on MRS agar plates (1.5 % agar) containing cellomixTM, 5 ㎍/㎖ erythromycin and 1 ng/㎖ nisin.

Especially, upon use of *Lactobacillus gasseri* as host cells, a total of 10 colonies which exhibit resistance to erythromycin and secrete cellulase, were isolated. Transformants secreting cellulases were screened by staining the colonies with a congo red solution for 30 min, followed by washing with 1 M NaCl until *cel*A halos appeared. The results are shown in Fig. 3.

Example 3

Determination of integration of pJC2 into *Lactobacillus* chromosomal DNA

3-1: PCR of *cel*A gene

To determine whether the integration vector pJC2 was integrated to chromosomal DNA of the transformed *Lactobacillus gasseri*, PCR was performed using chromosomal DNA of the transformed *Lactobacillus gasseri* as a template.

The chromosomal DNA of the transformed *Lactobacillus gasseri* was isolated by modification of a known method (O'sullivan, D.J. and T.R.Klaenhammer, *Applied. Environ. Microbiol.*, 59: 2730-2733, 1993). 1.5 ml of the *Lactobacillus* strain which had been cultured in MRS medium (Difco) was centrifuged at 4℃ and at 14, 000 rpm for 1 min, and the cells were collected. The collected cells were suspended in a 200 μℓ of TE buffer solution consisting of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). 60 μℓ of Lysozyme was added to the cell suspension and incubated at 37℃ for 30 min, thereby removing cell walls. The lysozyme-treated sample was added with 10 % SDS and proteinase K (20 mg/ml) and incubated at 37℃ for 1 hr. The proteinase-treated sample was then treated with phenol to remove proteins, and added with isoamylalcohol, followed by reaction at -20℃. The sample was centrifuged to precipitate DNA, thus isolating chromosomal DNA.

To perform PCR, the chromosomal DNA of *Lactobacillus gasseri* transformed with pJC2 thus isolated was used as a template. As primers, forward and reverse primers for *cel*A, that is, *cel*A-F and *cel*A-R, represented as SEQ ID NOS: 1 and 2, respectively, were used. The PCR conditions were 35 cycles with each cycle

consisting of 56℃, for 2 and a half hrs. As a positive control, the integration vector pJC2 alone as a template was subjected to PCR using the same conditions as in the above. The results are shown in Fig. 4. Lane 3 of Fig. 4 shows a band corresponding to a 1.4 kb *cel*A gene, indicating that the chromosomal DNA of *Lactobacillus gasseri* contains the pJC2 integration vector successfully integrated therein.

3-2: Southern blot using *cel*A gene probe

To confirm whether the integration vector pJC2 had been successfully integrated to chromosomal DNA of the transformed *Lactobacillus gasseri*, Southern blotting was performed using chromosomal DNA of the transformed *Lactobacillus gasseri*. As a negative control, chromosomal DNA of the non-transformed *Lactobacillus gasseri* was used. The chromosomal DNAs were isolated by the same method as in Example 3-1. Each of the two chromosomal DNAs was digested with *Sca*I, *Eco*RI and *Kpn*I. The digested fragments were separated by electrophoresis on a 0.8 % agarose gel. The DNA fragments on the agarose gel were transferred to a nitrocellulose membrane. Using an ECL direct nucleic acid labeling and detection system (Amersham Life Science), the 1.4 kb *cel*A PCR product obtained as in Example 3-1 was labeled and hybridized with the DNA fragments on the nitrocellulose membrane. As a negative control, the chromosomal DNA of the non-transformed *Lactobacillus gasseri* was subjected to Southern blotting, according to the same method as the above. As a positive control, the integration vector pJC2 itself was subjected to Southern blotting. The results are shown in Fig. 5. Lanes 2 and 3 of Fig. 5 indicate that the chromosomal DNA of *Lactobacillus gasseri* contains the pJC2 integration

vector successfully integrated therein.

Example 4

Construction of pJC7F, an integration vector containing ferritin gene for use in lactic acid bacteria

A synthetic promoter cp25 represented by SEQ ID NO: 3 and a 500 bp heavy chain gene of human ferritin was inserted to the integration vector pJC2 constructed as in Example 1. His · Tag was inserted between the cp25 promoter and the ferritin gene. Thus, an integration vector, pJC7F for use in lactic acid bacteria, was constructed. The cleavage map of pJC7F is shown in Fig. 6.

The integration vector pJC7F, for use in lactic acid bacteria, was deposited in an international depository authority, the Korean Culture Center of Microorganisms (KCCM), under deposit No. KCCM-10267 on May 14, 2001.

Example 5

Transformation using an integration vector, pJC7F and transformant screening

According to the same method as in Example 2, the integration vector pJC7F constructed as in Example 4 was used to transform *Lactobacillus gasseri* ATCC 33323. The transformed *Lactobacillus gasseri* was screened.

Example 6

Determination of integration of ferritin gene and expression thereof, and purification of ferritin protein

6-1: Determination of integration of ferritin gene

To determine whether the integration vector pJC7F has been integrated into chromosomal DNA of the transformed *Lactobacillus gasseri*, PCR and Southern blotting were performed by the same method as in Examples 3-1 and 3-2. Primers used in PCR were primers to the *cel*A gene, and primers represented by SEQ ID NO: 4, which are able to direct amplification of the promoter cp25 and the ferritin gene. The PCR conditions were 35 cycles with each cycle consisting of 58℃, for 2 hrs.

The PCR results are shown in Fig. 7. Lanes 2 and 3, using chromosomal DNA of the transformed *Lactobacillus gasseri* as a template, each show a 900 bp DNA band. The results indicate that the 900 bp DNA band corresponds to the band containing cp25 and ferritin genes on lane 5 of Fig. 7, using pJC7F as a template.

6-2: Determination of expressed ferritin protein and purification thereof

To determine whether *Lactobacillus gasseri* transformed with the integration vector pJC7F expresses a ferritin protein, the transformed *Lactobacillus gasseri* was cultured in 100 ml of MRS broth (Difco) containing 5 ㎍/㎖ erythromycin at 37℃ for 24 hrs. The cultured cells were centrifuged at 6,000 rpm for 5 to 7 min. After collecting the cells, they were suspended in a His · Tag binding buffer solution. The cells were disrupted by sonication once per sec. for 3 min at 20 % power in a sonicator (Sonics & Materials Inc.). Cell lysates were centrifuged at 6,000 rpm for 5 to 7 min. The supernatant was subject to a His · Bind Kit (Novagen) and His · Tag column (Novagen), thus purifying the ferritin protein.

The purified proteins were subjected to SDS-PAGE using a 12 % separating

gel and 5 % stacking gel, according to a known method. Thus, it could be seen that the transformed *Lactobacillus gasseri* expresses the ferritin protein, and it was found that the purified protein has a molecular weight of 23 kDa. Fig. 8 shows the results of SDS-PAGE of *Lactobacillus gasseri*. Compared to the cell extract of lane 8 in Fig. 8, lanes 2 and 7 referring to column eluents, which had passed through the His · Tag column, show partially purified ferritin proteins. When 14.4 ㎍/㎖ of total proteins was loaded on the His · Tag column, yield of the ferritin protein was 0.6 ㎍/㎖, the amount corresponding to 4.1 % relative to the total proteins.

Example 7

Transformation of *Lactobacillus johnsonii*

Transformation and confirmation thereof were performed by the same methods as in Examples 2 to 6, except *Lactobacillus johnsonii* NCK88 was used, instead of *Lactobacillus gasseri* ATCC 33323. The results of the Southern blot of the transformed *Lactobacillus johnsonii* using the *cel*A gene as a probe are shown in Fig. 5. Lanes 4 and 5 in Fig. 5 indicate that the integration vector pJC2 was successfully integrated into chromosomal DNA of the transformed *Lactobacillus johnsonii*.

Example 8

Transformation of *Lactobacillus bulgaricus*

Transformation and confirmation thereof were performed by the same methods as in Examples 2 to 6, except *Lactobacillus bulgaricus* was used, instead of *Lactobacillus gasseri* ATCC 33323. The results of the Southern blot of the

transformed *Lactobacillus bulgaricus* using the *cel*A gene as a probe are shown in Fig. 5. Lanes 8 and 9 in Fig. 5 indicate that the integration vector pJC2 was successfully integrated into chromosomal DNA of the transformed *Lactobacillus bulgaricus*. The results of PCR, where the cp25 promoter and ferritin genes were amplified using chromosomal DNA of *Lactobacillus bulgaricus* transformed with pJC7F as a template, are shown in Fig. 7. Lanes 2 and 3 each shows a 900 bp DNA band containing cp25 promoter and ferritin genes.

Further, transformation efficiencies of *Lactobacillus* strains transformed with the integration vector pJC2 according to the invention are shown in Table 1.

Table 1

Host cell	Transformation efficiency (colonies/μg DNA)
Lactobacillus gasseri	10
Lactobacillus johnsonii	10-15
Lactobacillus bulgaricus	30-40

As shown in Table 1, transformation efficiency was 10 colonies/μg DNA in *Lactobacillus gasseri*, 10-15 colonies/μg DNA in *Lactobacillus johnsonii*, and 30-40 colonies/μg DNA in *Lactobacillus bulgaricus*. Thus, the transformation efficiency with pJC2 of the invention was the highest in *Lactobacillus bulgaricus*.

Industrial Applicability

As apparent from the above description, the integration vector pJC2 for use in lactic acid bacteria was successfully integrated into chromosomal DNA of *Lactobacillus*, while the *Lactobacillus* transformed with the vector pJC7F successfully expressed a ferritin protein. Most of lactic acid bacteria secrete β-galactosidase, and frequently generate revertants upon use of an antibiotic resistance gene as a selection marker. For those reasons, there was a difficulty in screening only transformants on media containing X-gal and antibiotics. That problem can be solved by the use of a method of detecting a cellulase activity of bacteria transformed with the integration vector of the invention in which a *cel*A gene is inserted as a second selection marker gene. That is, with the integration vector of the invention, transformants can be easily screened. In addition, the integration vector of the invention can be integrated into the chromosomal DNA of a food grade lactic acid bacterium, thereby being successfully replicated therein. Accordingly, owing to the integration vector, a target protein can be successfully expressed in lactic acid bacteria. Moreover, the target protein expressed in lactic acid bacteria can be directly applicable as a food additive or pharmaceutical agent, without a step of removing possible toxicity.

Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM

OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
On page 19, lines 13.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on additional sheet ☒

Name of depositary institution

Korean Culture Center of Microorganisms(KCCM)

Address of depositary institution (including postal code and country)

Korean Culture Center of Microorganisms(KCCM)
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu
Seoul, 120-091, Republic of Korea

Date of deposit	Accession Number
May. 07, 2001	KCCM-10267

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information continues on an additional sheet ☒

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Claims:

1. A method for expressing ferritin in lactic acid bacteria transformed with a vector containing a ferritin gene.

2. The method according to claim 1, wherein the vector is a vector for use in lactic acid bacteria, containing an origin of replication.

3. The method according to claim 2, wherein the vector contains an origin of replication derived from a Gram-negative bacterium.

4. The method according to claim 1, wherein the vector is an integration vector containing an insertion sequence (IS) for use in lactic acid bacteria.

5. The method according to claim 4, wherein the insertion sequence is selected from the group consisting of IS1223, IS481, IS100 and IS903.

6. The method according to claim 1, wherein the vector contains a *cel*A gene for determining whether the vector is integrated into chromosomal DNA of a lactic acid bacterium.

7. The method according to claim 6, wherein the *cel*A gene has a nisin A promoter.

8. The method according to claim 1, wherein the vector contains an antibiotic resistance gene.

9. The method according to claim 8, wherein the antibiotic resistance gene is an erythromycin resistance gene or chloramphenicol resistance gene.

10. The method according to claim 1, wherein the ferritin gene has a promoter selected from the group consisting of a cp25 promoter, P21, P23 and P59.

11. The method according to claim 1, wherein the ferritin gene has a His · Tag sequence inserted between the ferritin gene and a promoter of the ferritin gene.

12. The method according to claim 1, wherein the vector is pJC7F, an integration vector for use in lactic acid bacteria, containing an origin of replication derived from a Gram-negative bacterium, an erythromycin resistance gene, a nisin A promoter, a *cel*A gene derived from *Clostridium thermocellum*, IS1223, a ferritin gene, a gene encoding His · Tag sequence, and a cp25 promoter represented by SEQ ID NO: 3 (deposit number: KCCM 10267).

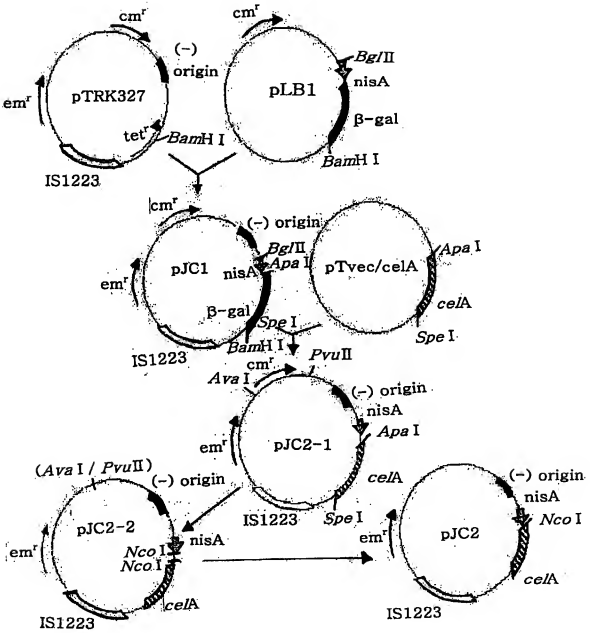
13. A food additive containing ferritin expressed by the method according to claim 1.

14. A pharmaceutical agent containing ferritin expressed by the method according to claim 1.

15. An integration vector for use in lactic acid bacteria, containing a *cel*A gene for determining whether the vector is integrated into chromosomal DNA of a lactic acid bacterium, and an insertion sequence.

16. An integration vector for use in lactic acid bacteria, pJC7F, containing an origin of replication derived from a Gram-negative bacterium, an erythromycin resistance gene, a nisin A promoter, a *cel*A gene derived from *Clostridium thermocellum*, IS1223, a ferritin gene, a gene encoding His · Tag sequence, and a cp25 promoter represented by SEQ ID NO: 3 (deposit number: KCCM 10267).

FIG. 1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nisA
ApaI
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pJC2-2
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pJC2

FIG. 2

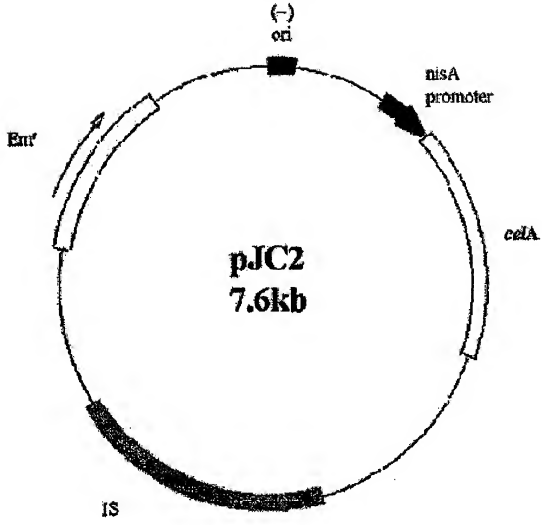


FIG. 3

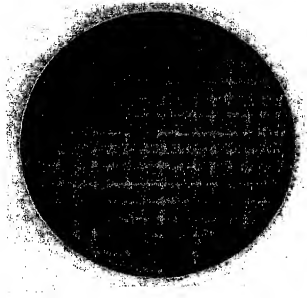


FIG. 4

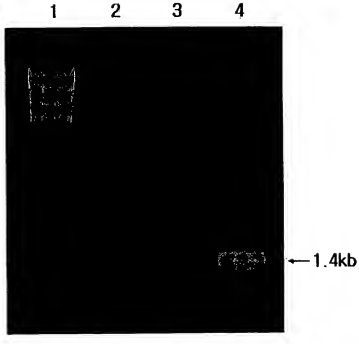


FIG. 5

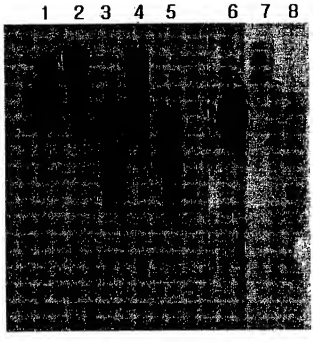


FIG. 6

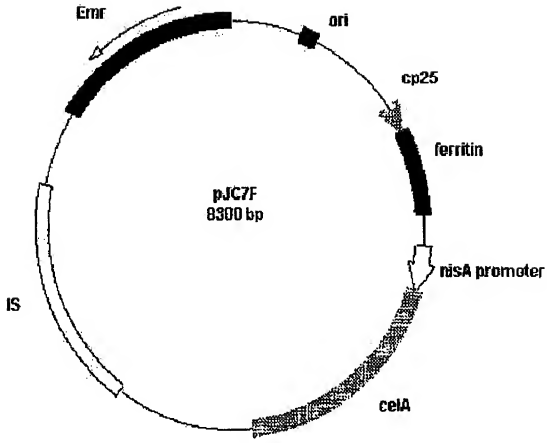


FIG. 7

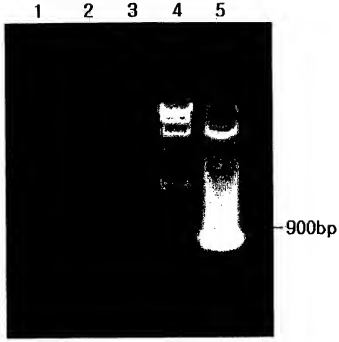
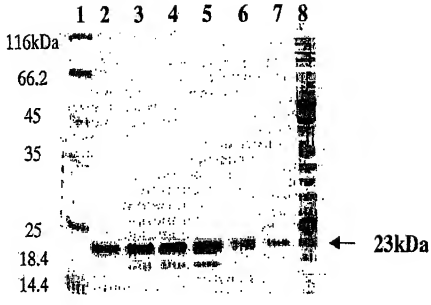


FIG. 8



Sequence Listing

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01452

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, PubMed, USPTO, PAJ, Espacenet, "ferritin", "lactic acid bacteria", "vector"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	US 5,580,787 (Stephen Wessels et al.), Dec. 3, 1996. see whole document	1-4, 8, 9 5-7, 10-12, 15, 16
Y A	WO 98/10079 A1 (Bioteknologisk Inst.), Mar. 12, 1998. see whole document	1-4, 8, 9 5-7, 10-12, 15, 16
Y A	Yi, X. et al., "Schistosoma Japonicum ferritin: Cloning, nucleotide sequencing, expression, and purification", Hunan Yike Daxue Xuebao, 23(5), 425-28, 1998. see abstract; & Chemical Abstract 130:234934	1-4, 8, 9 5-7, 10-12, 15, 16
Y A	Rucker, P. et al., "Recombinant ferritin: modulation of subunit stoichiometry in bacterial expression systems", Protein Eng., 10(8), 967-73, 1997. see abstract; & Chemical Abstract 128:125049	1-4, 8, 9 5-7, 10-12, 15, 16
X	Hayakawa, S., "Food material for iron supplement", Food Style 21, 4(3), 113-18, 2000. see abstract; & Chemical Abstract 133:119481	13
X	Cavina, G. et al., "Analytical and regulatory considerations for ferritin containing pharmaceutical products", Ann. Ist. Super. Sanita, 25(3), 471-79, 1989. see abstract; & Chemical Abstract 112:125312	14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 FEBRUARY 2002 (15.02.2002)	15 FEBRUARY 2002 (15.02.2002)
Name and mailing address of the ISA/KR Korean Intellectual Property Office Government Complex-Daejeon, 920 Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LEE, Cheo Young Telephone No. 82-42-481-5594

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01452

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	La Torre, F. et al., "Recent developments in the assay of ferritin containing drugs", Ann. Ist. Super. Sanita, 25(3), 481-85, 1989. see abstract; & Chemical Abstract 112:125313	14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR01/01452

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,580,787	Dec. 03, 1996	WO 91/09132	Jun. 27, 1991
WO 98/10079 A1	Mar. 12, 1998	US 6,133,023	Oct. 17, 2000